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THE USE OF TUMOR MODELS IN IMMUNOTOXICITY TESTING

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The role of the immune system in the prevention and control of tumor growth has been a rapidly expanding area of research during the past decade. The concept that a host could immunologically recognize and thus eliminate transformed cells from the body offered many new prospects for the prevention and treatment of cancer. From a toxicology standpoint, however, immunologic control of tumor growth presented an additional area for concern - that is, that even if a chemical was not directly carcinogenic, it might, through effects on the immune system, create an individual that was more susceptible to tumor growth, and thus present an even more subtle hazard than a direct carcinogen.

Because of the biologic and toxicologic relevance of a tumor model, our laboratory has been working to develop suitable tumor models for use in immunotoxicity testing. In addition to the toxicologic significance of altered tumor susceptibility due to chemical exposure, we felt that a tumor model could also provide several testing advantages from a technical standpoint. The first major advantage of a tumor model is the availability of multiple in vivo endpoints for assessing altered host susceptibility. These include tumor frequency, tumor latency and growth rate, progression versus regression, and metastases development. The availability of more than a single all-or-nothing endpoint such as mortality should provide increased sensitivity for detecting immunologic alterations due to chemical exposure.

The second major advantage of a tumor model is the ability to quantitate essentially all major aspects of the immune response within a single system. These include the surveillance mechanisms of natural killer (NK) cells and macrophages which control susceptibility to tumor growth as well as specific antitumor immune responses which control tumor growth rate and perhaps metastases development. Specific antitumor host defenses include cytolytic T cells, cytolytic and cytostatic macrophages, complement-dependent cytotoxic antibodies, antibody-dependent K cell cytotoxicity, and lymphokine production. Furthermore, in a tumor model one can also examine the immunologic mechanisms whereby tumors can escape from antitumor defenses, including the level of serum blocking factors and suppressor cell activity.

The third major advantage of a tumor model is that all the responses are quantitated in terms of a common antigenic stimulus. Using the tumor cells as antigen provides a common basis by which all the in vitro functional assays can be compared and, more important, correlated to the phenomenon of in vivo tumor growth.

Several tumor systems have been used in immunotoxicity assessments (Table 1). The majority are mouse tumors and their use has been limited to the study of in vivo host susceptibility. Both virus and chemically induced tumors, as well as spontaneous tumor models have been utilized. However, a lack of interlaboratory comparison of sensitivities makes it difficult to recommend one model over another. Advantages of each model may depend on the experimental conditions. For example, lung tumor models may be of greatest relevance in inhalation exposure studies. The ability of the tumor to metastasize may also be a relevant factor in some studies.

TABLE 1. TUMOR MODELS USED IN IMMUNOTOXICITY ASSESSMENT

Tumor	Strain of Origin	Endpoints	Reference
MKSA (SV-40 virus)	BALB/c	Tumor frequency, latency, volume	Dean et ai., 1979
PYB6 (polyoma virus)	C57BL/6	Tumor frequency, latency, volume	Dean et al., 1980
MSV-MSB (Moloney sarcoma virus)	C57BL/6	Tumor frequency, progression, secondary challenge	Kerkvliet et al., 1979, 1980, 1982a,b
RLV (Rauscher leukemia virus)	C57BL/6	Leukemia and death	Gainer, 1972
MOPC-104	BALB/c	Time to death	Bellanti et al., 1978
Walker carcinosarcoma	Sprague-Dawley	Tumor frequency, latency, volume, metastases	Kerkvliet and Kimeldorf, 1977
Lewis lung tumor	BALB/c	Lung tumor nodules	Dean et al., 1980
B16F10 melanoma	C57BL/6	Lung tumor nodules	Dean et al., 1982

Three mouse tumor models have been primarily utilized in our laboratory for the study of chemical-induced immunotoxicity. Their sensitivity in detecting immune alterations induced by exposure to pentachlorophenol (PCP), a widely used wood preservative, will be described (Kerkvliet et al., 1982a). In these studies, C57BL/6 female mice, 8 weeks of age, were placed on diets contaminated with 50 or 500 ppm pure (99+%) or technical (86%) grade PCP. The diets were available to the animals ad libitum for 8 weeks prior to tumor challenge. The commercially available technical PCP is known to be contaminated with significant levels of other chlorinated phenols as well as chlorinated dibenzofurans and dioxins.

The first tumor model assesses the ability of the animals to resist challenge with a syngeneic tumor given at a low tumor dose, producing a 10-30% incidence of progressive tumors in normal, untreated mice. Low dose tumor challenge is considered a sensitive assessment of general immunocompetence, with tumor resistance likely representing the surveillance mechanisms of NK cells and/or macrophages. We used a methylcholanthrene-induced sarcoma of C57BL/6 mice injected subcutaneously at a dose of 1×10^4 tumor cells. Mice were examined three times per week for appearance of tumors, and, once palpable, tumor growth rates were estimated by caliper measurements of the tumor diameters.

Exposure of mice to technical grade PCP resulted in a significant increase in susceptibility to low dose tumor challenge (Table 2). The incidence of progressive tumor growth increased from 35% in control mice to 67 and 82% in animals exposed to 50 and 500 ppm technical PCP, respectively. Animals exposed to pure PCP at the same dietary levels did not show any significant alteration in tumor susceptibility with a tumor incidence of 31 and 40% in the 50 and 500 ppm exposure groups, respectively. The effect of technical PCP was observed only at the level of initial susceptibility; the growth rates of the tumors that developed and host survival time were not significantly different from controls.

TABLE 2. EFFECT OF PCP EXPOSURE ON SUSCEPTIBILITY OF MICE TO LOW-DOSE TUMOR CHALLENGE[a]

Treatment ppm PCP	Progressive Tumor Incidence[b] (%)
0	9/26 (35)
50 pure	4/13 (31)
500 pure	6/15 (40)
Dose Response (P)	NS
50 technical	10/15 (67)
500 technical	9/11 (82)[c]
Dose Response (P)	<0.005

a Mice were injected with 1×10^4 Sarcoma 1412 cells s.c. and were observed twice weekly for 90 days.

b Number of mice with tumor/number of mice injected.

c Significantly different from 0 ppm, χ^2, $p < 0.05$.

The second tumor model that we have utilized most extensively is the Moloney sarcoma virus (MSV)-induced tumor system in C57BL/6 mice. The MSV system is an attractive model for immunotoxicity assessments because of the predictable and short induction time of strongly antigenic tumors which leads to spontaneous tumor regression in immunocompetent hosts (Levy and Leclerc, 1977). Tumors induced by MSV appear in 5-10 days at the site of virus injection, reach a peak size around day 14, and completely regress by day 21. The mechanisms of tumor regression have been well-studied, with tumor regression primarily dependent on an intact cytotoxic T lymphocyte response. However, growth inhibitory macrophages (Holden et al., 1976; Korn et al., 1978a,b), cytotoxic antibodies (Leclerc et al., 1972; Lamon et al., 1973), and antibody-dependent K cell cytotoxicity (Pollack, 1973; Pollack et al., 1972) also appear to play a role in MSV tumor regression. Furthermore, animals that have undergone primary MSV tumor growth and regression retain specific antitumor immunity (Holden et al., 1975), rendering the animals resistant to a secondary challenge with MSV-transformed tumor cells (MSB) injected at a dose that produces a 100% incidence of progressive tumors in non-MSV immune animals. Thus, the MSV model is useful for the examination of toxicant effects on both primary and secondary antitumor immune responses.

The applicability of the MSV system to secondary challenge has proven to be a very useful aspect of the model as it appears to provide increased sensitivity in detecting immunosuppression induced by chemical exposure. In studies to further assess the immune suppression induced by PCP exposure, mice were exposed to diets contaminated with 50 or 500 ppm pure or technical PCP. After 8 weeks of exposure, the mice were inoculated intramuscularly in the left hind leg with MSV. Primary tumor growth and regression was monitored daily, and, after 2 months, all regressor animals were reinoculated in the right hind leg with 1×10^6 MSB tumor cells. The animals were monitored for an additional 2 months for appearance of MSB tumors, after which time all survivors were killed and necropsied.

Results of this study are summarized in Table 3. Following MSV injection, all control and PCP-exposed animals developed primary tumors at the site of virus inoculation with peak tumor size observed on days 9-10 followed by tumor regression. However, on approximately day 18, 55% of the animals exposed to 500 ppm technical PCP exhibited a recurrence of tumor growth which progressed until the death of the host. Complete tumor regression occurred in all other groups. Following secondary challenge with MSB, the incidence of progressive tumor growth was significantly elevated in animals exposed to 50 ppm technical PCP; two of five animals exposed to 500 ppm technical PCP that survived the initial MSV challenge also developed progressive MSB tumors. The combined mortality from primary and secondary tumor challenges was thus increased from 19% in controls to 45 and 73% in animals exposed to 50 and 500 ppm technical PCP, respectively. As with the low-dose tumor challenge model, animals exposed to pure PCP did not show enhanced MSV-MSB tumor susceptibility.

TABLE 3. EFFECT OF PCP EXPOSURE ON SUSCEPTIBILITY OF MICE TO PROGRESSIVE PRIMARY MSV-INDUCED TUMOR GROWTH AND RESISTANCE TO SECONDARY MSB CHALLENGE[a]

Treatment ppm PCP	Progressive Tumor Incidence (%) Primary MSV	Secondary MSB[b]	Total MSV/MSB
0	0/16 (0)	3/16 (19)	3/16 (19)
50 pure	0/10 (0)	1/10 (10)	1/10 (10)
500 pure	0/11 (0)	2/11 (18)	2/11 (18)
Dose Response (P)			NS
50 technical	0/11 (0)	5/11 (45)	5/11 (45)
500 technical	6/11 (55)[c]	2/5 (40)	8/11 (87)[c]
Dose Response (P)			<0.005

a Mice were injected with MSV in the right hind leg; primary tumor growth and regression/progression were monitored for 105 days. Regressor animals were reinoculated in the left hind leg with 1 x 10^6 MSB cells. Secondary tumor growth was monitored for an additional 50 days.

b Six of six non-MSV immunized mice inoculated with 1 x 10^6 MSB cells died from progressive tumor growth.

c Significantly different from 0 ppm, χ^2, $p < 0.05$.

However, when animals that were resistant to both MSV and MSB challenges were necropsied, sarcoma development was unexpectedly observed in the spleen of several PCP-exposed animals. The incidence of splenic tumors was 50% (3/6) in animals exposed to 50 ppm technical PCP, 22% (2/9) in animals exposed to 50 ppm pure PCP, and 44% (4/9) in animals exposed to 500 ppm pure PCP. Splenic tumor formation was not observed in any of the 13 surviving control animals nor in the 3 animals remaining in the 500 ppm technical PCP group. The development of splenic tumors following MSV injection is a phenomenon that we have not previously observed in normal animals during our 4 years of work with the MSV system. However, metastases of MSV to the spleen has been reported to occur in immunoincompetent newborn mice (Perk and Moloney, 1966) and in adult mice immunosuppressed by x-irradiation or neonatal thymectomy (Stanton et al., 1968). These results suggest that splenic tumor development may provide a highly sensitive parameter for detecting immune suppression induced by exposure to environmental chemicals.

The third tumor model that has been used in our laboratory for assessing chemical-induced immunotoxicity is an allograft model, the DBA/2 (H-2^d) P815 mastocytoma transplanted into C57BL/6 (H-2^d) mice. This model has been used primarily for the in vitro assessment of tumor immunity. Allogeneic sensitization of test animals by a single intraperitoneal injection of tumor cells results in a strong immune reaction directed against the histocompatibility antigens on the tumor cells. This immune response is expressed in high levels of cytotoxic T cell activity, high cytotoxic antibody titers, and activation of the mononuclear phagocyte system. All these responses can be readily measured in a single animal, using spleen cells for T cell cytotoxic activity, serum for antibody titration, and peritoneal exudate cells for macrophage activation. In addition, under circumstances of severe immunodepression, the in vivo growth of the allogeneic tumor can be used as a host susceptibility assay. The P815 tumor model also offers several advantages from a technical standpoint since quantitation of all the in vitro assays is based on the efficient and objective measurement of radioactive label release or uptake. T cell cytotoxicity, for example, is easily measured by the in vitro lysis of ^{51}chromium-labelled tumor cells (Brunner et al., 1970). The methodology involves the labelling of the P815 tumor cells with ^{51}Cr followed by a 4-hour incubation of the labelled tumor cells with serial dilutions of spleen cells that were obtained from animals injected 10 days previously with 1 x 10^7 P815 tumor cells. During incubation the cytotoxic T cells present in the spleen cell suspension lyse the tumor cells causing the release of ^{51}Cr into the medium. Following centrifugation, an aliquot of the cell-free supernatant is harvested and the amount of radioactivity present is quantitated on a gamma scintillation counter. The amount of ^{51}Cr released is then plotted against the ratio of spleen cells to tumor cells assayed. Cytotoxic activity can then be expressed in terms of lytic units (LU) where 1 LU is equal to the number of spleen cells causing 50% lysis of the tumor cells. Based on the number of spleen cells recovered per animal, the number of LU per spleen can be calculated. This same ^{51}Cr release assay can be used to quantitate antibody and macrophage cytotoxicity, simply by substituting serially diluted serum (in the presence of complement) or peritoneal cells for the spleen cells.

Thus far we have only utilized the cytotoxic T cell assay in immunotoxicity studies of PCP, with the assays for cytotoxic antibody and macrophages under development. As shown in Figure 1, animals exposed to technical PCP contaminated diets exhibited an exposure level-dependent reduction in splenic T cell cytotoxicity. The reduction was statistically significant at all effector:target cell ratios in the 500 ppm technical PCP group. No change in lytic activity was apparent using cells from mice exposed to pure PCP. The similarity in the slopes of the curves suggested that there was no qualitative difference in lytic activity between the cytotoxic cells from technical PCP-exposed and control animals, but rather a reduction in the percentage of cytotoxic cells present in the spleen of technical PCP-exposed mice. Thus, as shown in Table 4, on a LU basis, essentially twice as many spleen cells were required from the 500 ppm technical PCP exposed mice to lyse the same number of tumor cells as control. Coupled with a reduction in the total number of spleen cells recovered from the 500 ppm technical PCP exposed animals, a 55% reduction in the number of LU/spleen was observed. These results directly support the previous findings of enhanced tumor susceptibility observed in vivo in technical PCP-exposed animals, and suggest that the mechanism of enhanced tumor growth may be due to a reduction in T cell cytotoxicity induced by exposure to PCP.

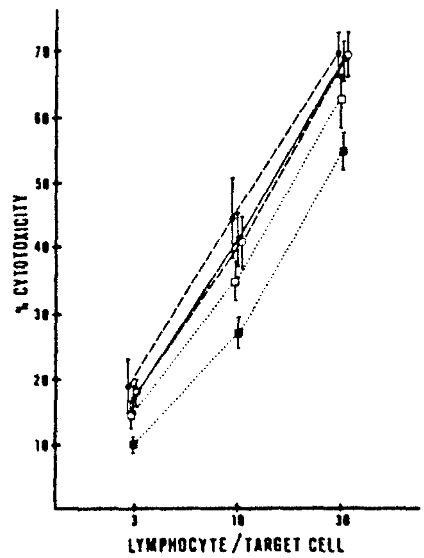

Figure 1. Effect of PCP exposure on T cell-mediated cytotoxic activity of spleen cells from P815-allogeneic sensitized mice. Cytotoxicity determined in a 6 hour ^{51}Cr-release assay using P815 mastocytoma cells as targets. Data represent mean ± SE of the cytotoxic response of 4-6 animals tested individually. X ——— control, o --- 50 ppm pure PCP, ● ---- 500 ppm pure PCP, ☐ ······ 50 ppm technical PCP, ■ ······ 500 ppm technical PCP.

TABLE 4. EFFECT OF PCP EXPOSURE ON CYTOTOXIC T CELL ACTIVITY[a]

Treatment ppm PCP	LU_{50}[b] ($\times 10^{-4}$)	Spleen Cell[c] Recovery ($\times 10^{-7}$)	LU/Spleen (% of Control)
0	12.5 ± 1.7	13.5 ± 0.6	$11,284 \pm 1,264$
50 pure	13.0 ± 2.7	11.7 ± 0.8	$10,112 \pm 1,227$ (90)
500 pure	12.4 ± 1.7	12.0 ± 0.5	$10,689 \pm 1,818$ (95)
50 technical	17.3 ± 4.0	14.4 ± 1.4	$9,823 \pm 2,424$ (87)
500 technical	24.0 ± 3.3[d]	10.8 ± 0.8[d]	$5,102 \pm 1,018$[d] (45)

[a] Values presented as mean $\pm$ se, 4-5 animals/group.

[b] LU_{50} defined as the number of spleen cells required to lyse 50% of 1×10^4 ^{51}Cr-labelled P815 tumor cells in 6 hours.

[c] Spleen cell recovery after 10 sec. hypotonic lysis of RBCs; viability >90% in all groups.

[d] $P < 0.05$, Student's t test.

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